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## Authors

Lima, Bruno
Hu, Linda
Vreeman, Gerrit
et al.

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# The oral bacterium *Fusobacterium nucleatum* binds *Staphylococcus aureus* and alters expression of the staphylococcal accessory regulator *sarA*

**Bruno P. Lima**[#,a,b], **Linda I. Hu**[#,c,d], **Gerrit W. Vreeman**[b], **Douglas B. Weibel**[c,e,f], **Renate Lux**[*,a]

[a]Division of Constitutive and Regenerative Sciences, University of California, School of Dentistry, Los Angeles, CA.

[b]Present address: Department of Diagnostic and Biological Sciences, School of Dentistry, University of Minnesota, Minneapolis, MN.

[c]Department of Biochemistry, University of Wisconsin-Madison, 440 Henry Mall, Madison, WI.

[d]Present address: Department of Microbiology-Immunology, Feinberg School of Medicine, Northwestern University, Chicago, IL.

[e]Department of Chemistry, University of Wisconsin-Madison, 440 Henry Mall, Madison, WI.

[f]Department of Biomedical Engineering, University of Wisconsin-Madison, 440 Henry Mall, Madison, WI

## Abstract

*Staphylococcus aureus*, an opportunistic pathogen member of the nasal and skin microbiota, can also be found in human oral samples and has been linked to infectious diseases of the oral cavity. As the nasal and oral cavities are anatomically connected, it is currently unclear whether *S. aureus* can colonize the oral cavity, and become part of the oral microbiota, or if its presence in the oral cavity is simply transient. To start addressing this question, we assessed *S. aureus* ability to directly bind selected members of the oral microbiota as well as its ability to integrate into a human-derived complex oral microbial community *in vitro*. Our data show that *S. aureus* forms aggregates with *Fusobacterium nucleatum* and *Porphyromonas gingivalis* and that it can incorporate into the human-derived *in vitro* oral community. Further analysis of the *F. nucleatum*-*S. aureus* interaction revealed that the outer-membrane adhesin RadD is partially involved in aggregate formation and that the RadD-mediated interaction leads to an increase in expression of the staphylococcal global regulator gene *sarA*. Our findings lend support to the notion that *S. aureus* can become part of the complex microbiota of the human mouth, which could serve as a reservoir for *S. aureus*.. Furthermore, direct interaction with key members of the oral microbiota could affect *S. aureus* pathogenicity contributing to the development of several *S. aureus* associated oral infections.

[*]To whom correspondence may be addressed: Renate Lux, Division of Constitutive and Regenerative Sciences, University of California School of Dentistry, Los Angeles, CA, USA. Tel.: (310) 206-5660, rlux@dentistry.ucla.edu.

[#]These authors contributed equally

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### Keywords

oral ecology; Staphylococcus; Fusobacterium; coaggregation; SarA

## Introduction

*Staphylococcus aureus* is an opportunistic pathogen most commonly isolated from the human anterior nares (i.e., nostrils) and skin. Equipped with a wide range of virulence factors, *S. aureus* has been associated with several infectious diseases, including pneumonia, septicemia, and endocarditis [1]. In addition to the human anterior nares and skin, a recent retrospective analysis of ten years of laboratory data reported that *S. aureus*—including some methicillin-resistant *S. aureus* (MRSA) strains—was isolated from approximately 20% of oral and perioral samples, leading to the proposition that the human oral cavity serves as a reservoir for *S. aureus* [2].

The anterior nares could serve as a possible source of the *S. aureus* isolated from the human oral cavity, as these two regions are anatomically connected and nasal staphylococci share a high level of genetic relatedness to oral staphylococci [3]. In the oral cavity, *S. aureus* has been linked to several infectious diseases, including: angular cheilitis [4], osteomyelitis of the jaw [5], parotitis [6], periodontitis [7], peri-implantitis [8], and endodontic infections [9–11]. While disease-associated *S. aureus* colonization of the oral cavity is well-established, the underlying mechanisms for *S. aureus* colonization are not.

The oral cavity is home to hundreds of bacterial species that associate with each other through a complex network of interactions which often involve physical binding. These interactions contribute to the formation of a sophisticated multispecies ecosystem known as the oral biofilm. Similar to many complex microbial communities, the oral microbial community can resist colonization by exogenous organisms [12–14], presumably helping prevent dysbiosis and contributing to human health. It remains unclear whether *S. aureus* can colonize the oral cavity and become part of the oral microbial community or whether its presence among oral bacteria is transient due to the anatomic connection between the nasal and oral cavities.

In this paper, we start to address the question whether *S. aureus* has the ability to colonize the human oral cavity by determining its ability to directly bind selected members of this community, including *Fusobacterium nucleatum, Porphyromonas gingivalis* as well as several gram-positive early colonizers. We determined that both the lab strain (SH1000) as well as a methicillin-resistant strain of *S. aureus* (USA300) bind and form aggregates with *F. nucleatum* and *P. gingivalis* but not the other oral species tested, *in vitro*. We also show that interaction with *F. nucleatum* affects *S. aureus* gene expression.

Although the oral microbiota is robust and uses colonization resistance to sidestep bacterial invaders [15,14], our data demonstrate that *S. aureus* can integrate into a human-derived, mixed-species oral microbial community *in vitro* for at least 6 days and that its presence alters the community composition. These findings lend credence to hypothesis that the human oral cavity may serve as a reservoir for *S. aureus*.

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## Materials and methods

### Bacteria and culture conditions

All bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise stated, *F. nucleatum* strains were grown in columbia broth or on columbia agar plates (BD Difco, Detroit, MI) supplemented with 5% defibrinated sheep blood (Colorado Serum Company, Denver, CO), anaerobically (5% $H_2$, 5% $CO_2$, 90% $N_2$) at 37 °C. *P. gingivalis* was also grown anaerobically in columbia broth supplemented with 5% defibrinated sheep blood containing hemin and menadione at 5 μg/mL and 1 μg/mL, respectively. *Streptococcus sanguinis* and *S. gordonii* were grown anaerobically in Todd Hewitt (TH) broth or agar plates (BD Difco, Detroit MI) at 37 °C. *Actinomyces naeslundii* ATCC 12104 was grown in Brain Heart Infusion (BHI) broth or agar plates (BD Difco, Detroit MI) at 37 °C also anaerobically. *S. aureus* was grown in tryptic soy broth (TSB) or on tryptic soy agar (BD Difco, Detroit, MI) in anaerobic conditions or 66% TSB (TSB66) in aerobic conditions. As a model for the human-derived, complex oral microbiota, we used a previously described cultivable oral microbial community [16,17] grown in SHI medium [17] anaerobically at 37 °C.

### Saliva collection and preparation

Stimulated whole saliva was collected from six healthy adult volunteers in compliance with the Institutional Review Boards from the University of California Los Angeles (IRB#11–002483). Volunteers collected 30–40 mL of saliva (stored on ice) after refraining from consuming food and brushing their teeth for at least 2 hours. After collection, the saliva was combined in equal volumes and pelleted at 6000 × *g* for 10 min at 25 °C. Saliva was diluted two-fold with water and filter sterilized using a 0.22 μm membrane (Merck Millipore Ltd, Tullagreen, Carrigtwohill, Co. Cork, Ireland).

### Coaggregation assays

Coaggregations were performed in coaggregation buffer (CAB) (150 mM NaCl, 1 mM Tris, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$) as previously described [18] with minor modifications. Overnight cultures of *S. aureus and F. nucleatum* were diluted in the morning and grown until they reached late exponential growth phase. Bacterial cells were harvested by centrifugation for 2 min at 13000 × *g*, washed with CAB and resuspended in CAB. Equal numbers of bacterial cells were combined to a final concentration of $2 \times 10^9$ cells/mL in 400 μL of CAB in a 1.5 mL microcentrifuge tube. The tubes were vortexed for 10 s and incubated for 10 min at 25 °C. Coaggregation was scored on a 0–4 scale as described previously [19] or quantified using an spectrophotomer as follows: After incubation, the bacterial mixtures were centrifuged at low speed (100 × *g*) for 1 min to pellet co-aggregating cells, while leaving the non-aggregated bacteria in suspension. Two hundred microliters of the supernatant were removed without disturbing the pellet and the optical density of the recovered supernatant was measured at $\lambda$=600 nm. For coaggregation inhibition assays, L-arginine, D-galactose, D-glucose or lactose were added to coaggregation reactions to a final concentration of 50 mM. Relative coaggregation was quantified by subtracting the turbidity of the recovered supernatant after coaggregation (ODpost) from the turbidity of the

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cell mixture before coaggregation (ODpre) and dividing the result by the turbidity before coaggregation (ODpre) [(ODpre - ODpost) / ODpre] [20]

## Biofilm growth

S. aureus *mono-species biofilms in TSB66 medium.* Strains were grown on TSB agar plates overnight at 37 °C. Single-colony cultures in liquid TSB medium were grown overnight with shaking (200 rpm) at 37 °C. Overnight cultures were diluted 1:1000 in TSB66 that was supplemented with either sterile saliva, starch, or maltose. This diluted culture of 195 μL was transferred into 96-well polystyrene plates, sealed with Breathe Easy Sealing adhesive membrane, and grown with shaking at 200 rpm at 37 °C overnight. Unattached cells were removed, and the wells were gently rinsed with 150 μL phosphate-buffered saline (PBS). Adherent cells and extracellular materials were stained with 100 μL of 0.5 % w/v crystal violet solution for 10 min at 25°C and washed 3 times with 150 μL PBS. Plates were dried for 20–45 min, during which they were imaged. One hundred microliters of acidified ethanol (4% v/v of 1 M HCl in ethanol) was added and incubated for 5 min at 25 °C. The solubilized crystal violet was transferred into a clean 96-well plate and read at λ=570 nm.

F. nucleatum *mono-species and dual-species biofilms with* S. aureus. Five hundred μL of SHI-FS [50% SHI medium [17], 25% filtered saliva] containing $1\times10^8$ *F. nucleatum* cells (and $5\times10^4$ *S. aureus* for dual-species biofilms) cells diluted from overnight cultures were added to the wells of a 48-well polystyrene culture plates (Thermo Fisher Scientific, Waltham, MA, USA) and incubated overnight under anaerobic conditions (5% $H_2$, 5% $CO_2$, 90% $N_2$) at 37 °C. After overnight growth, planktonic cells were removed, and the biofilm was washed 3 times with 500 μL of pre-reduced, sterile PBS. The biofilm that remained attached to the wells was stained with 500 μL of 0.5% crystal violet solution for 10 min at 25 °C and then washed 3 times with sterile PBS to remove excess crystal violet. After the final PBS wash, 500 μL of acidified ethanol was added to the wells and incubated for 10 min at 25°C to dissociate crystal violet from the biofilm. Crystal violet retention was measured spectrophotometrically at λ=570 nm.

## Human-derived multispecies oral microbial community biofilm.

The the human-derived mutispecies oral microbial community was grown overnight in SHI medium [17] and diluted in the morning to an absorbance of 0.1 (λ=600 nm) in fresh SHI medium. *S. aureus* was inoculated at an absorbance of 0.01 (λ=600 nm). Biofilms were grown using 24-well polystyrene culture plates (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 6 days under anaerobic conditions (5% $H_2$, 5% $CO_2$, 90% $N_2$) at 37°C. Samples were collected at days 0, 2, 4, and 6. Except for the samples collected at day 0, the planktonic cells were removed from the wells and the biofilms were washed 3 times with 500 μL of pre-reduced, sterile PBS. Biofilms that remained attached to the wells after washing were collected for DNA isolation.

## Nucleic acid isolation, PCR and denaturing gradient gel electrophoresis (DGGE).

Genomic DNA was extracted from biofilms using the MasterPure™ DNA Purification Kit (Epicenter®, Madison, WI, USA) according to manufacturer's instructions. The concentration of purified bacterial DNA was determined by Nanodrop 2000 (Thermo

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Scientific, Waltham, MA, USA). Primers Bac1 with a GC clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAC TAC GTG CCA GCA GCC-3') and Bac2 (5' -GGA CTA CCA GGG TAT CTA ATC C-3') [21] were used to amplify a region approximately 300-base-pair in length (bp) of the 16S ribosomal RNA gene. PCR amplification was confirmed by gel electrophoresis in a 1.0% agarose gel. Polyacrylamide gels (8%) were prepared with a denaturing urea/formamide gradient ranging from 40% to 60%. Approximately 45 µL of the PCR product were loaded into each well. The gel was submerged in TAE buffer (40 mM Tris base, 40 mM glacial acetic acid, 1 mM ethylenediaminetetraacetic acid) and the PCR products were separated by electrophoresis for 17 h at 58 °C using a fixed voltage of 60 V in a Bio-Rad DCode System (Bio-Rad Laboratories, Inc., Hercules, CA). Gels were stained with ethidium bromide to visualize the DNA bands in the gel. Gel images were taken with the Molecular Imager Gel Documentation system (Bio-Rad Laboratories). All experiments were performed in triplicate for each time point.

Total RNA was extracted using PureLinK™ RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. Genomic DNA contamination was removed from total RNA using Turbo DNA-free™ Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions and confirmed by PCR using 16S rRNA primers Bac1 and Bac2 [21].

### qPCR

The *S. aureus*-specific primers Sa083F and Sa083R [22] were used to quantify *S. aureus* DNA in mixed biofilm populations. The 16S rRNA universal primers BAC1 and BAC2 were used to quantify the total DNA collected from the biofilms. qPCR was performed using an iCycler Thermal Cycler (Bio-Rad, Hercules, CA) in a total volume of 20 µL containing 2 µL of 10x iQ SYB® Green Supermix (Bio-Rad, Hercules, CA), 0.5 µM each of forward and reverse primers, 7 µL of Millipore water, and 1 µL (10ng) of template DNA. Amplification and detection were carried out in 96-well optical plates (Thermo Fisher Scientific, Waltham, MA). Each PCR run was carried out with an initial incubation of 10 min at 95 °C followed by 40 cycles of denaturing at 95 °C for 15 s; annealing and elongation at 60 °C for 1 min. After amplification, an additional denaturing step was performed at 95 °C for 1 min followed by annealing and elongation at 60 °C for 1 min. A melting curve analysis was completed after each run. DNA concentrations (ng/mL) were calculated with standard curves obtained by ten-fold serial dilutions of bacterial genomic DNA. Three independent qPCR runs were performed with three technical replicates for each sample to assess reproducibility and inter-run variability.

### RT-qPCR

To determine the expression pattern of virulence-factor-associated genes, 1 µg of total RNA was used for cDNA synthesis using SuperScript® III First-Strand (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's protocol. For qRT-PCR, iQ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) was used for fluorescence detection with the iCycler real-time PCR system (Bio-Rad) according to the manufacturer's instructions. The DNA gyrase gene *gyrR* was used as internal standard for expression analysis. Expression levels of *gyrR* were determined using

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5′- CATTGCCAGATGTTCGTGAC –3′ forward and 5′- CCGGTGTCATACCTTGTTCA –3′ reverse primers for normalization of the qRT-PCR data [23]. The *sarA* transcript was amplified using 5′- TCTTGTTAATGCACAACGTAA –3′ forward and 5′- TGTTTGCTTCAGTGATTCGTTT –3′ reverse primers [23]; *agr* was amplified using 5′- CCAGTTTGCCACGTATCTTC –3′ forward and 5′- TGCATCCCTAAACGTACTTGC –3′ reverse primers [23].

### 16S rRNA gene sequencing

The microbial composition was determined using next-generation sequencing in conjunction with a customized Basic Local Alignment Search Tool (BLAST) program [ProbeSeq for Human Oral Microbe Identification using Next Generation Sequencing (HOMINGS), Forsyth Institute, Boston, MA] for bacterial analysis. ProbeSeq contains sequences of genus-specific as well as species-specific custom made 16S rDNA probes (17–40 bases) based on the Human Oral Microbe Database (HOMD) [24], most of which were originally developed for the Human Oral Microbe Identification Microarray (HOMIM) [25]. Briefly, a fragment of the 16S rRNA gene sequence located between variable regions V3 and V4 was amplified using broad range primers: 341F (5’-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT ATG GTA ATT GTC CTA CGG GAG GCA GCA G-3’) and 806R (5’-CAA GCA GAA GAC GGC ATA CGA GAT NNN NNN NNN NNN AGT CAG TCA GCC GGA CTA CHV GGG TWT CTA AT-3’). After amplification, the DNA sequences were purified using AMPure beads. Next, a library of 100 ng of DNA was pooled, gel-purified, and subsequently quantified by quantitative PCR (qPCR). Finally, 12 pM of the library was added to 20% Phix and sequenced using MiSeq (Illumina, San Diego, CA). At least 200,000 reads were obtained per sample.

### Statistical analysis

Student’s *t*-test [Microsoft® Excel version 16.13.1 (Microsoft, Seattle, WA, USA)] or one-way ANOVA (http://turner.faculty.swau.edu/mathematics/math241/materials/anova/) were performed to determine statistical significance.

## Results

### *S. aureus* forms biofilm in starch and saliva-supplemented growth medium

In the oral environment, microbial biofilms form in the presence of saliva. Thus, we tested whether saliva affected *S. aureus* biofilm formation. Increasing concentrations of pooled, filter-sterilized saliva (0–12.5% v/v final concentrations) was added to TSB66, a nutrient medium used previously to study *S. aureus* biofilms [26]. While the addition of filter-sterilized saliva to TSB66 medium slightly reduced biofilm formation by *S. aureus* strain SH1000 as measured using a crystal violet retention assay of biofilms 20 h post-inoculation (Fig. 1A), the addition of 2% w/v starch to TSB medium supplemented with 12.5% saliva led to a ~50% increased crystal violet retention (Fig. 1B). This starch-induced increase in biofilms is more pronounced at higher concentrations of saliva according to an one-way comparison between treatments by ANOVA (10% saliva [$F(4,10)=14.87$, $p=0.0003$] and 12.5% saliva [$F(4,10)=3.95$, $p=0.0354$]) (Fig. 1C). No significant amount of crystal violet was detected in wells that did not contain *S. aureus* (Fig. S1)

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In the oral cavity, starch is quickly hydrolyzed into maltose by amylase present in saliva [27]. We supplemented TSB66 with maltose and observed a dose-dependent increase in biofilm formation compared to cells grown in TSB66 without maltose (Fig. S2A). It is unlikely that the increase in biofilm is due to the presence of an easily fermentable carbon source since no growth advantage was observed by the addition of maltose to the the media (Fig. S2B). Instead, the optical density from the planktonic phase decreased with increasing concentration of maltose (Fig. S2A), which may suggest that maltose induces surface attachment.

### *S. aureus* bind to *F. nucleatum*

In addition to biofilm formation, we investigated if *S. aureus* cells directly bind oral bacteria. We screened a panel of oral bacterial species including the early colonizers: *Streptococcus gordonii* DL1, *Streptococcus sanguinis* SK36, and *Actinomyces naeslundii* ATCC12104; and the late colonizers and periodontal pathogens: *F. nucleatum* ATCC23726, *P. gingivalis* 4612, for their ability to bind *S. aureus* strain SH1000 as well as the MRSA strain USA300 *in vitro* in coaggregation experiments. Both strains of *S. aureus* formed large aggregates (coaggregation score of 4) with *F. nucleatum* and smaller aggregates (coaggregation score of 2) with *P. gingivalis* (Table 2). No coaggregation was detected between *S. aureus* cells and the other oral species tested, highlighting the specificity of the interaction between *S. aureus* and *F. nucleatum* as well as *P. gingivalis*. As the coaggregation phenotype was stronger with *F. nucleatum* than with *P. gingivalis*, we focused our investigation in the identification of the molecular components involved in the cell-cell interaction between *S. aureus* and *F. nucleatum*. Additionally, since no difference was observed between the two strains of *S. aureus* in their ability to bind to *F. nucleatum*, all subsequent experiments were performed using the laboratory strain SH1000 rather than the MRSA derivative USA300.

### Characterization of the *S. aureus-F. nucleatum* interaction

*F. nucleatum* is an important member of the oral microbiota and adheres to many different bacterial species within the oral microbial community [28]. Some of these cell-cell interactions can be inhibited by L-arginine [18,29–31], while D-galactose and D-lactose have been shown to inhibit others [32,33]. To characterize the physical interaction between *S. aureus* and *F. nucleatum*, we added L-arginine, D-galactose, D-glucose, or D-lactose (all to a final concentration of 50 mM) to coaggregation buffer as reported previously [32]. Coaggregation between *F. nucleatum* and *S. aureus* cells was reduced by 80% with the addition of 50 mM L-arginine but was unaffected by the presence of the same concentration of D-galactose, D-glucose, or D-lactose (Fig. 2A). These results implicate an arginine-inhibited adhesion mechanism in the *S. aureus-F. nucleatum* interaction.

We previously identified and inactivated six *F. nucleatum* auto-transporter-like outer membrane proteins (OMPs) that bind L-arginine: Fn0254, RadD (Fn1526), CmpA (Fn1554), Fn1893, Fn2047, and Aim1 (Fn2058) [18]. Of those, RadD and CmpA have been shown to be involved in coaggregation [34,20] and are referred to as adhesins. As L-arginine inhibited coaggregation between *F. nucleatum* and *S. aureus* cells (Fig. 2A), we hypothesized that the *S. aureus-F. nucleatum* interaction involved at least one of these six arginine-binding OMPs. To test this hypothesis, we screened each of the previously constructed *F. nucleatum* OMP

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mutants for their ability to coaggregate with *S. aureus*. Lack of Fn0254, CmpA, Fn1893, Fn2047, and Aim1 had no effect on coaggregation, however, inactivation of *radD* decreased coaggregation between *F. nucleatum* and *S. aureus* cells by ~35% (Fig. 2B), implicating RadD in this interaction.

### *S. aureus-F. nucleatum* dual-species biofilms

Surface attachment and biofilm formation are crucial for microbial persistence and survival in the oral cavity [35]. Previous studies demonstrate that *F. nucleatum* utilizes the surface protein RadD to bind and form dual-species biofilm with other oral species [18,32,20]. To further characterize the potential role of RadD-mediated interaction between *S. aureus* and *F. nucleatum* in dual-species-biofilm formation, we co-incubated *S. aureus* with *F. nucleatum* (wild-type or *radD* mutant) for 24 h in 50% SHI medium supplemented with 25% saliva [which has been shown to support the growth of a multispecies oral community [36]] and measured early stages of biofilm formation using crystal-violet-retention assay. Inoculation of *F. nucleatum* or *S. aureus* alone resulted in the formation of a thin layer of biofilm, as detected by crystal violet; coinoculation of both organisms (*S. aureus: F.nucleatum*) enhanced biofilm formation by ~13 and 3-fold compared to *F. nucleatum* or *S. aureus* alone, respectively (Fig. 3). A mutant of *F. nucleatum* lacking RadD reduced dual-species biofilm formation to the level of *S. aureus* alone, suggesting that the increase in biofilm formation when *F. nucleatum* and *S. aureus* are co-cultured occurs in a RadD-dependent manner (Fig. 3).

### *F. nucleatum* induces expression of the virulence-factor-associated *sarA* in *S. aureus*.

Bacteria often respond to the presence of other species by altering gene and/or protein expression [for a review see [37]]. To determine if the interaction with *F. nucleatum* influences *S. aureus* physiology through transcriptional changes, we monitored the expression of two known global regulators in *S. aureus*: the accessory gene regulator (*agr*); and the staphylococcal accessory regulator (*sarA*). Similarly to a study assessing the effect of *S. gordonii* on *P. gingivalis* physiology [38], we combined an equal number of wild-type *S. aureus* and *F. nucleatum* cells in liquid culture, the cells were pelleted to maximize direct cell contact, and co-incubated for 6 h in anaerobic conditions. Expression of *sarA* and *agrB* was measured by RT-qPCR. The presence of *F. nucleatum* resulted in a ~5-fold increase in *sarA* transcription compared to the absence of *F. nucleatum*, while no difference in transcription was observed for *agrB* (Fig. 4A). No change in *sarA* nor *agrB* transcription occurred when *S. aureus* was co-incubated with *S. gordonii* (**data not shown**), suggesting that the *F. nucleatum* effect in *sarA* transcription is not a general response of *S. aureus* to the presence of another an oral microbe. Since *radD* inactivation affects coaggregation and dual-species biofilm formation (Fig. 2B and 3, respectively), we tested if RadD was required for the increase in *sarA* expression. Co-incubation with the *F. nucleatum radD* mutant did not induce *sarA* transcription (Fig. 4B).

### *S. aureus* cells integrate into multispecies oral bacterial biofilms

The observations that *S. aureus* binds to *F. nucleatum* cells and responds physiologically (Figs. 3 and 4) led us to investigate whether *S. aureus* cells could persist in a complex

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multispecies oral biofilm community. To explore this concept, we utilized a previously published human-derived *in vitro* oral multispecies community containing a bacterial phylotype diversity representative of dental plaque [16,17]. We grew this human-derived oral community *in vitro* in anaerobic conditions in SHI media for six days with or without *S. aureus*. We collected biofilms at days 0, 2, 4, and 6 and confirmed the presence of *S. aureus* DNA in the community by PCR using *S. aureus*-specific primers against the *gltA* gene [22]. These primers have been shown to be able to detect as little as 10 copies of *S. aureus* DNA. We determined the total DNA concentration of the biofilm by qPCR of the 16S rRNA gene and found that the concentration of total DNA, as well as the concentration of *S. aureus* DNA, as determined by qPCR, increased over time (Fig. 5A and 5B), suggesting that *S. aureus* can grow within a complex oral microbial community.

### *S. aureus* affects the composition of the human-derived, multispecies oral community

In complex microbial communities, bacteria constantly share, exchange, and compete for resources, which can influence community composition. As *S. aureus* incorporates into our *in vitro* oral microbial community, we tested if the presence of *S. aureus* had any impact in the overall community composition. To address this point, we used degenerate primers Bac1 and Bac2 [21] to amplified the 16S rRNA gene from total DNA collected from the mixed oral community grown with and without either strains of *S. aureus* and performed a DGGE analysis. Community profiling via DGGE revealed that the presence of either SH1000 or USA300 *S. aureus* had a similar influence on oral microbial community composition compared to samples that were grown without *S. aureus* (Fig. 6A). Next generation sequencing of the V3 and V4 regions of the 16S rRNA gene derived from the samples above revealed that in the absence of *S. aureus*, streptococci are the most abundant phylum in this oral community. After co-incubation with *S. aureus*, the composition of the community shifts to one in which staphylococci are predominant (Fig. 6B).

## Discussion

*S. aureus* is an important opportunistic human pathogen, which is generally accepted as a resident of the skin and nasal microbiota. However, *S. aureus* has also been identified by some studies as a potentially important player in peri-implantitis and aggressive periodontitis [8,39,7]. Despite the anatomical connection between the nasal and oral niches, the status of *S. aureus* as an oral microbe is controversial, despite its implication in certain oral diseases [3]. Here, we provide data that could help explain how *S. aureus* could colonize the oral cavity. Our data demonstrate that *S. aureus* cells can grow and form biofilms under *in vitro* conditions that have characteristics of the oral cavity, in particular, growth in the presence of saliva and starch. We also show that *S. aureus* binds directly to members of the oral microbial community and incorporates into a human-derived complex oral microbial community *in vitro*, affecting community composition. Specific interaction with the oral bacterium *F. nucleatum* results in differential expression of the *S. aureus* global regulator *sarA*, which is connected to the attenuation of pathogenicity, biofilm formation and susceptibility to antibiotic treatment [40–42]. Thus, this study supports that *S. aureus* is capable of establish itself in the human oral community *in vitro*, and if reproduced *in*

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*vivo* we think the oral cavity could serve as a reservoir for this opportunistic pathogen and potentially influence its virulence behavior through modulation of the global regulator *sarA*.

## Interaction between *F. nucleatum* and *S. aureus* cells involves *F. nucleatum* RadD

Direct cell-cell adhesion has been studied for many oral bacterial species [28]. *F. nucleatum* is regarded as an important and prevalent oral species that is able to bind to numerous bacterial species in the oral cavity [28]. This ability is thought to enable *F. nucleatum* to serve as an anchor for species that are unable to attach directly to oral surfaces and/or early biofilm formers, such as streptococci [43–45]. Our data demonstrate that *S. aureus* cells form mixed-species aggregates with *F.* nucleatum and to a lesser extend with *P. gingivalis* which could implicate *F. nucleatum* as a primary anchoring point for *S. aureus* integration into the oral community. *F. nucleatum* expresses several surface adhesin some of which are known to mediate its attachments to other microbial genera and species within the oral community. Among these proteins, RadD is regarded as a major adhesin that influences the interaction between *F. nucleatum* and *S. sanguinis, S. mutans, S. gordonii*, *Streptococcus oralis, A. naeslundii*, some strains of *P. gingivalis*, as well as *Candida albicans* [32,18,46]. Our data indicate that RadD is one of the adhesins involved in the interaction between *S. aureus* and *F. nucleatum*; however, RadD is unlikely to be the sole adhesin involved in the interaction between these two bacteria, as lack of RadD did not fully abolish coaggregation between *F. nucleatum* and *S. aureus*. This is similar to our previous findings that the interaction of *F. nucleatum* with several strains of *S. gordonii* and *P. gingivalis* is mediated by RadD in combination with additional adhesins [32,20]. Our screen of five *F. nucleatum* adhesin mutants did not identify additional adhesins involved in the *F. nucleatum-S. aureus* cell interaction, which supports the existence of other arginine-binding adhesins that has not yet been identified. Similar results have been observed for the *F. nucleatum-P. gingivalis* interaction [32].

## Implications for a selective oral environment

A series of well-orchestrated interactions establish and maintain complex polymicrobial communities. These interactions ultimately help maintain an optimal bacterial population which fights off invading species, some of which could be harmful to the host {for a review, please see [37]}. As one of the primary point of access to the digestive tract, one would expect that the oral cavity would also contain numerous species of enteric bacteria; however, this does not seem to be the case [47]. One explanation for the inability of enteric bacteria to colonize the oral cavity is the release of inhibitory substances, such as $H_2O_2$, which can be toxic to some species [15]. Interestingly, *S. aureus* was able to escape such defense mechanism in our *in vitro* system and was able to incorporate and grow in the biofilm formed by the oral community (Fig. 5), even though it is sensitive to $H_2O_2$ (**data not shown**). This ability to incorporate into a complex oral community, we think, could make the human oral cavity a reservoir for *S. aureus*.

## Synergy between *F. nucleatum* and *S. aureus* cells

Many human infections are polymicrobial and can be aggravated by the interaction between species, which is a phenomenon referred to as ‘microbial synergy’ [48–50]. In fact,

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microbial profiling of severe acute oral abscesses identified *F. nucleatum* and *S. aureus* as common members of the microbial consortia associated with this disease [51,52], suggesting that these niches may provide one location where synergistic interactions may occur. We provide a possible outcome to this putative synergistic interaction; our *in vitro* data demonstrate that the *F. nucleatum-S. aureus* interaction increases biofilm formation (Fig. 3) and leads to increased expression of the global regulator *sarA* (Fig. 4). SarA has been shown to play an important role in the expression of many *S. aureus* virulence factors, including toxins, adhesins, and biofilm formation [53–56], thus, interaction with *F. nucleatum* has the potential to exacerbate *S. aureus* pathogenicity.

## Concluding Remarks:

In summary, we determined that *S. aureus* can grow and form biofilms in *in vitro* conditions that mimic the oral environment. We identified *F. nucleatum* as a binding partner for *S. aureus*, which could enable its integration into oral biofilm communities. We further characterized RadD as one of the fusobacterial adhesins involved in allowing *S. aureus* to establish itself in this community, which is known to be resilient against invaders. We found that the physical contact between *F. nucleatum* and *S. aureus* affects gene expression, in a RadD-dependent manner, of an important global regulator in *S. aureus*, *sarA*, which has potential implications for virulence gene expression. Identification of factors allowing *S. aureus* establishment in body sites such as the oral cavity, which can serve as a reservoir for focal infections, is important for addressing the larger medical problem that this species comprises for certain patient populations.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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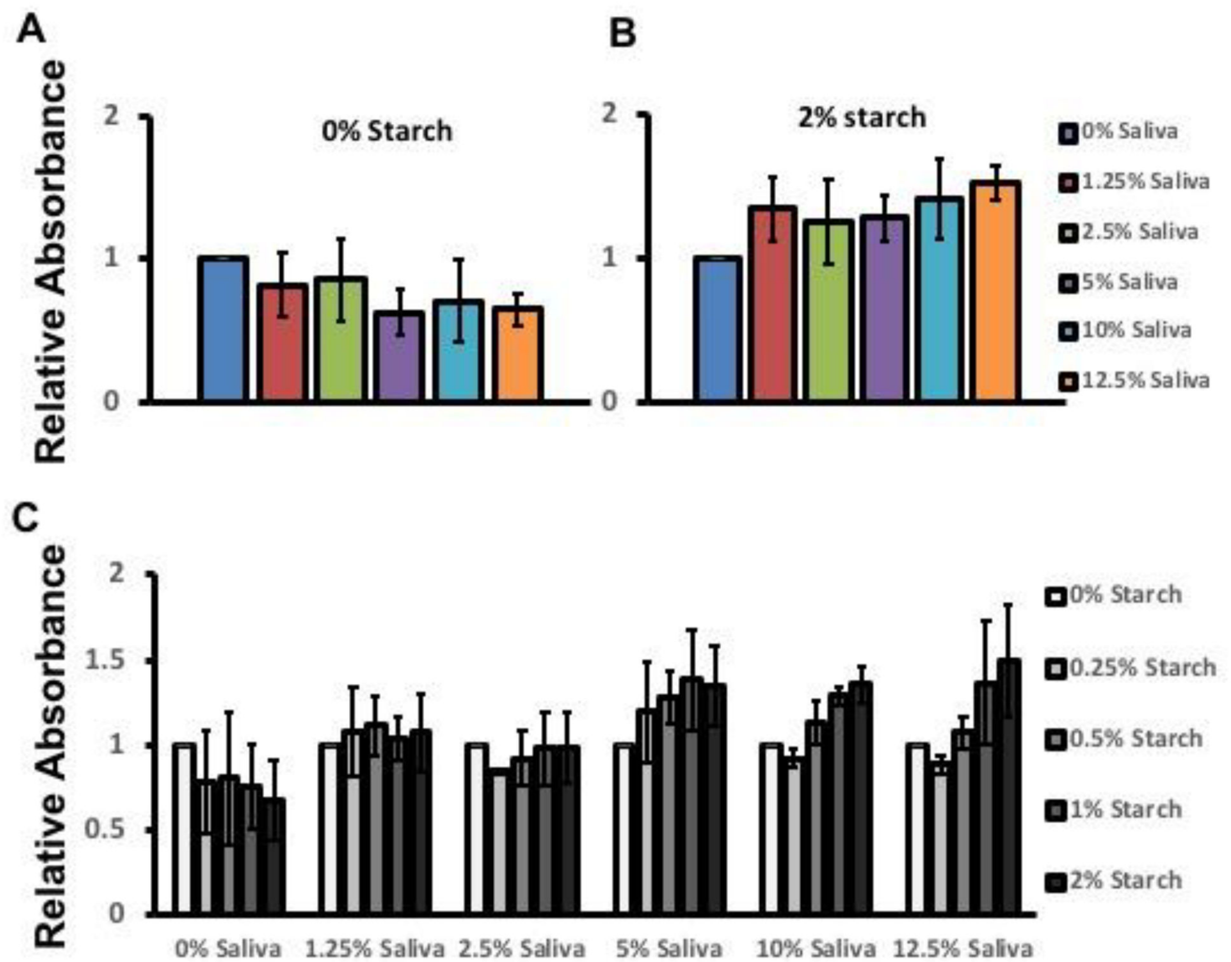


**Fig. 1).**

A mixture of saliva and starch or maltose in growth medium induces biofilm formation in *S. aureus* SH1000. *S. aureus* SH1000 was grown in a 96 well plate in filter-sterilized TSB66 containing 0–12.5% v/v saliva and 0–2% starch w/v in combination or alone shaking with aeration for 24 h. Attached cells were stained with crystal violet. **(A, B)** Crystal violet retention from three independent experiments were quantified, averaged, and plotted relative to 0% saliva, or **(C)** 0% Starch.

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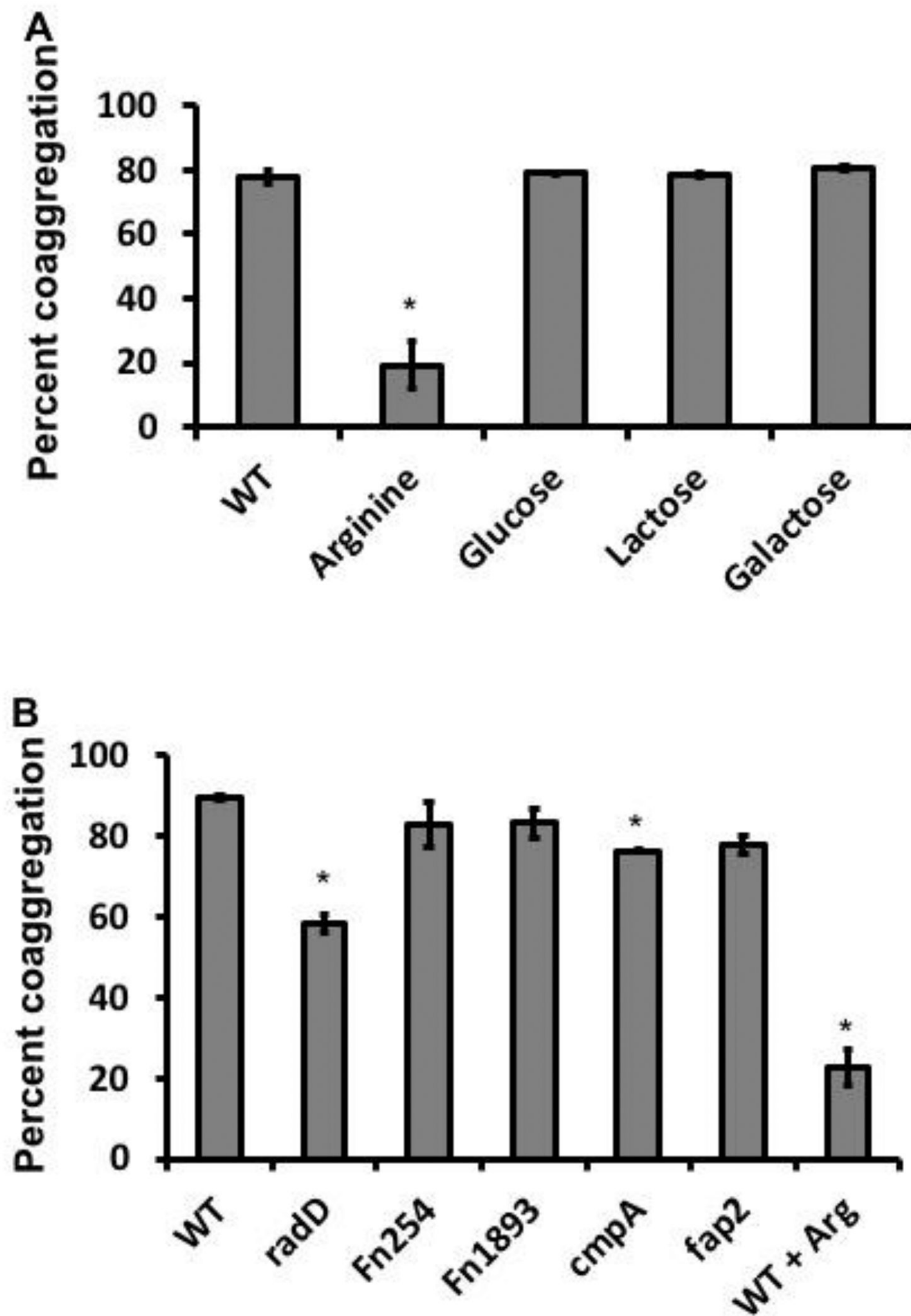


**Fig. 2).**

Quantitative coaggregation of **(A)** wildtype *S. aureus* SH1000 with the wildtype (WT) *F. nucleatum* strain ATCC 23726, or the (WT) *F. nucleatum* in the presence of 50 mM arginine, glucose, lactose, or galactose. **(B)** Quantitative coaggregation of wildtype *S. aureus* SH1000 with the wildtype (WT) *F. nucleatum* strain ATCC 23726 (+/− 50 mM Arginine (Arg) or the *F. nucleatum* mutant derivatives (*radD*, Fn0254, Fn1893, *cmpA* and *fap2*). Data represent means and standard deviation of percent coaggregation of at least three independent experiments.

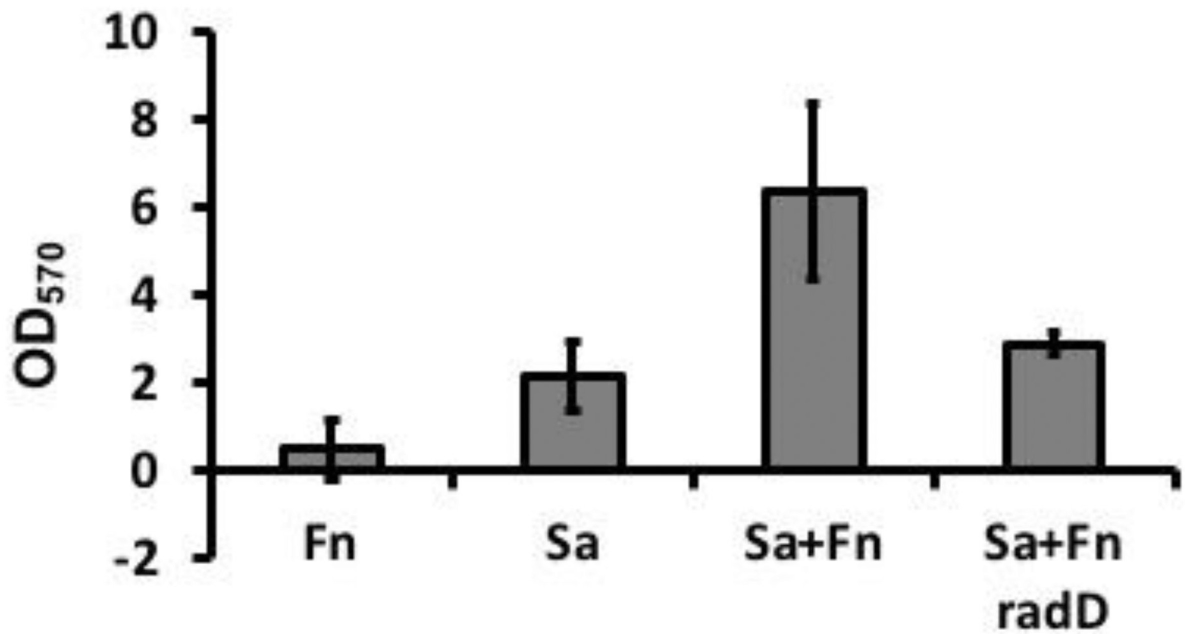


**Fig. 3).**

Biofilm formation of *F. nucleatum* (Fn) and *S. aureus* SH1000 (Sa) cells, as well as Sa with WT Fn and a *radD* mutant. Strains were grown anaerobically at 37°C for 16 h. Crystal violet retention was used as an indicator of biomass. Data represent means and standard deviation of percent coaggregation of at least three independent experiments.



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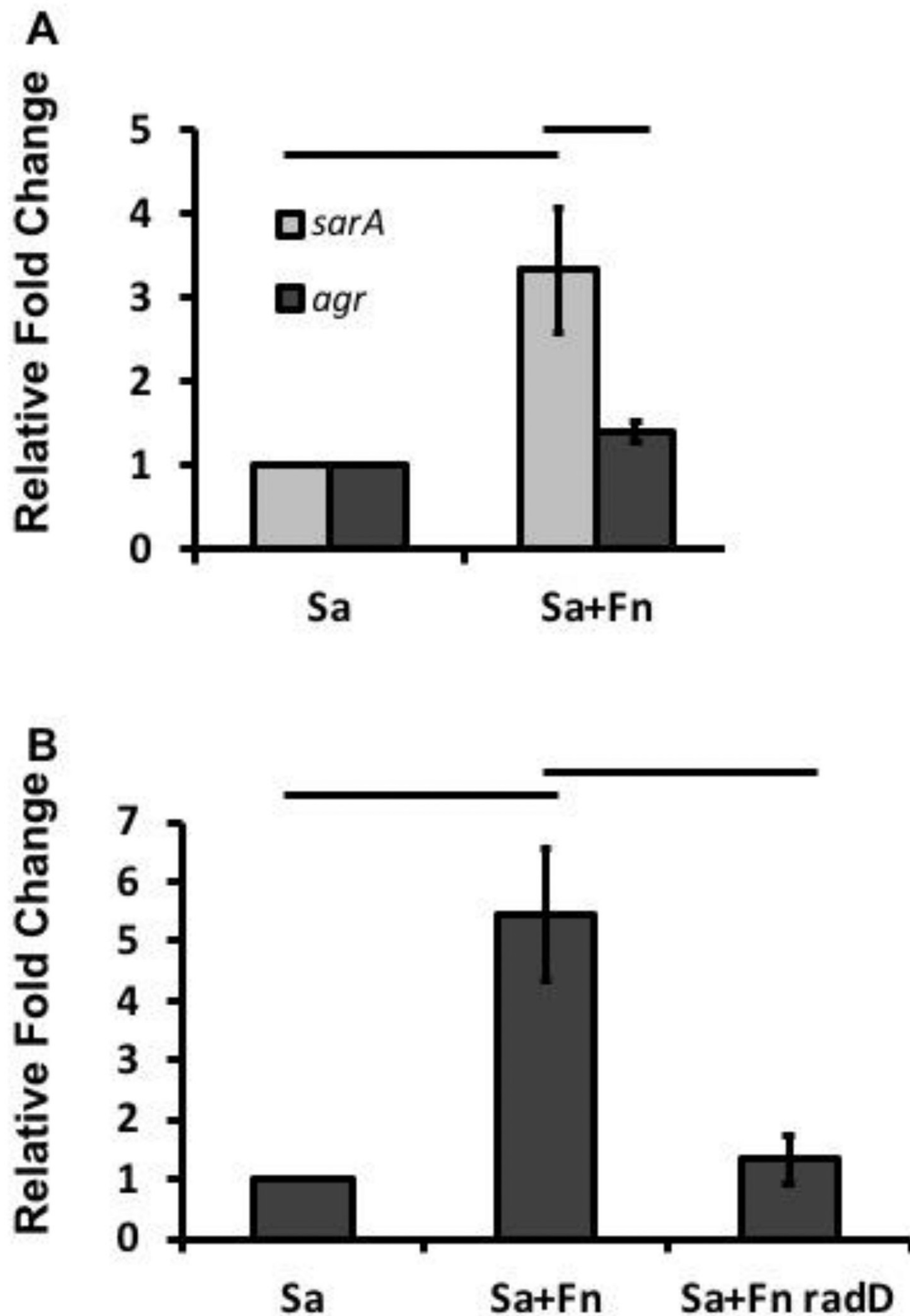


**Fig. 4).**

*sarA* and *agr* expression: mid-log-phase cells of *F. nucleatum* (WT or the *radD* mutant), and *S. aureus* grown in Columbia or TSB, respectively, were combined for 6 h anaerobically. qRT-PCR was used to determine **(A)** relative *sarA* and *agr* expression in the presence of WT *F. nucleatum* compared to *S. aureus*. **(B)** Relative *sarA* expression in the presence of WT *F. nucleatum* or the radD mutant derivative compared to *S. aureus*. Each value represents means and standard deviation of at least three independent experiments. To aid with visualization, the dashed line represents no change.

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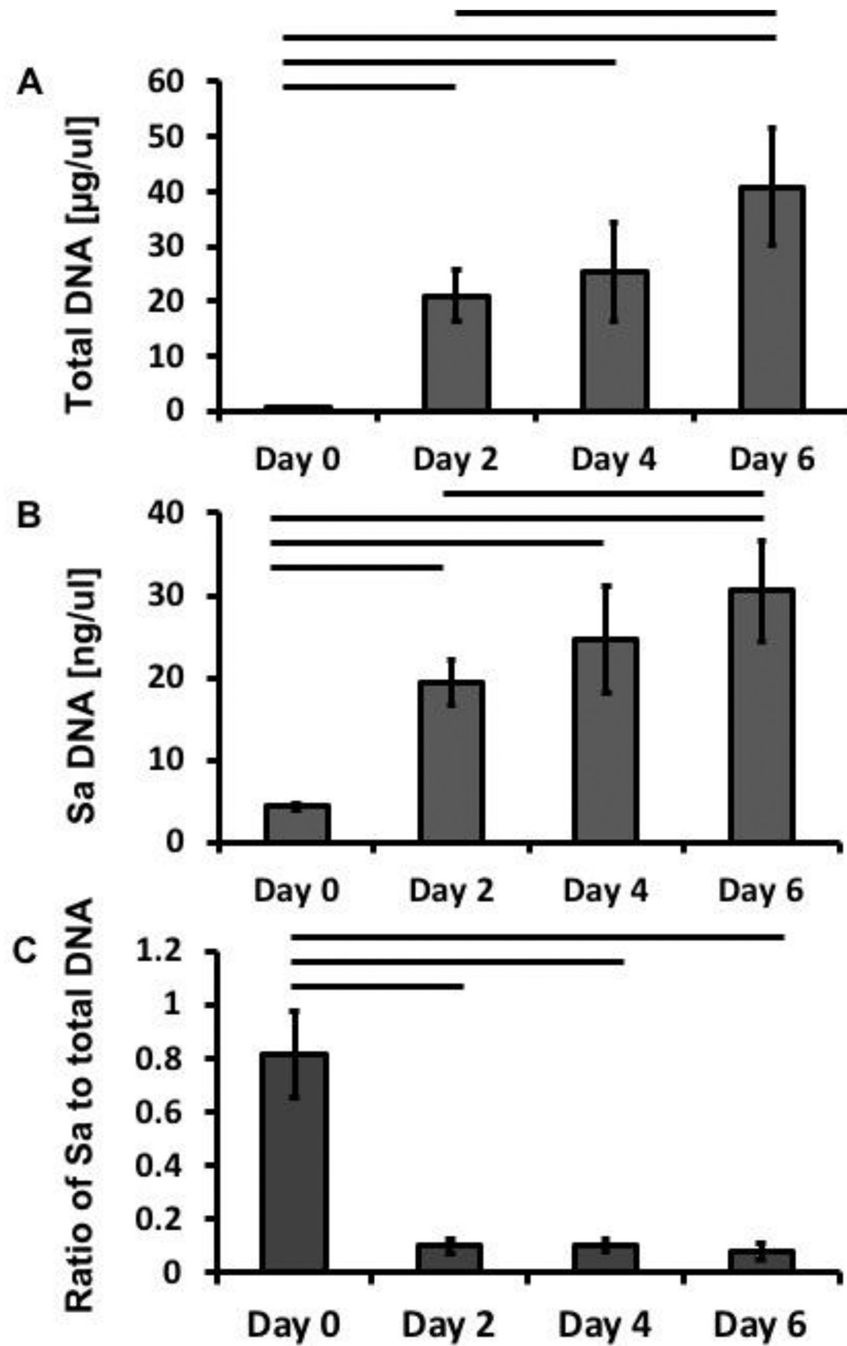


**Fig. 5).**

DNA concentration over time. DNA was collected at days 0, 2, 4 and 6 and measured by qPCR. **(A)** total bacterial DNA concentration was determined by amplifying the 16s rRNA gene. **(B)** *S. aureus* DNA concentration was determined using *S. aureus* specific primers to amplifying the *gltA* gene. **(C)** Ratio of *S. aureus* to total DNA. The values represent means and standard deviation of three biological replicates.

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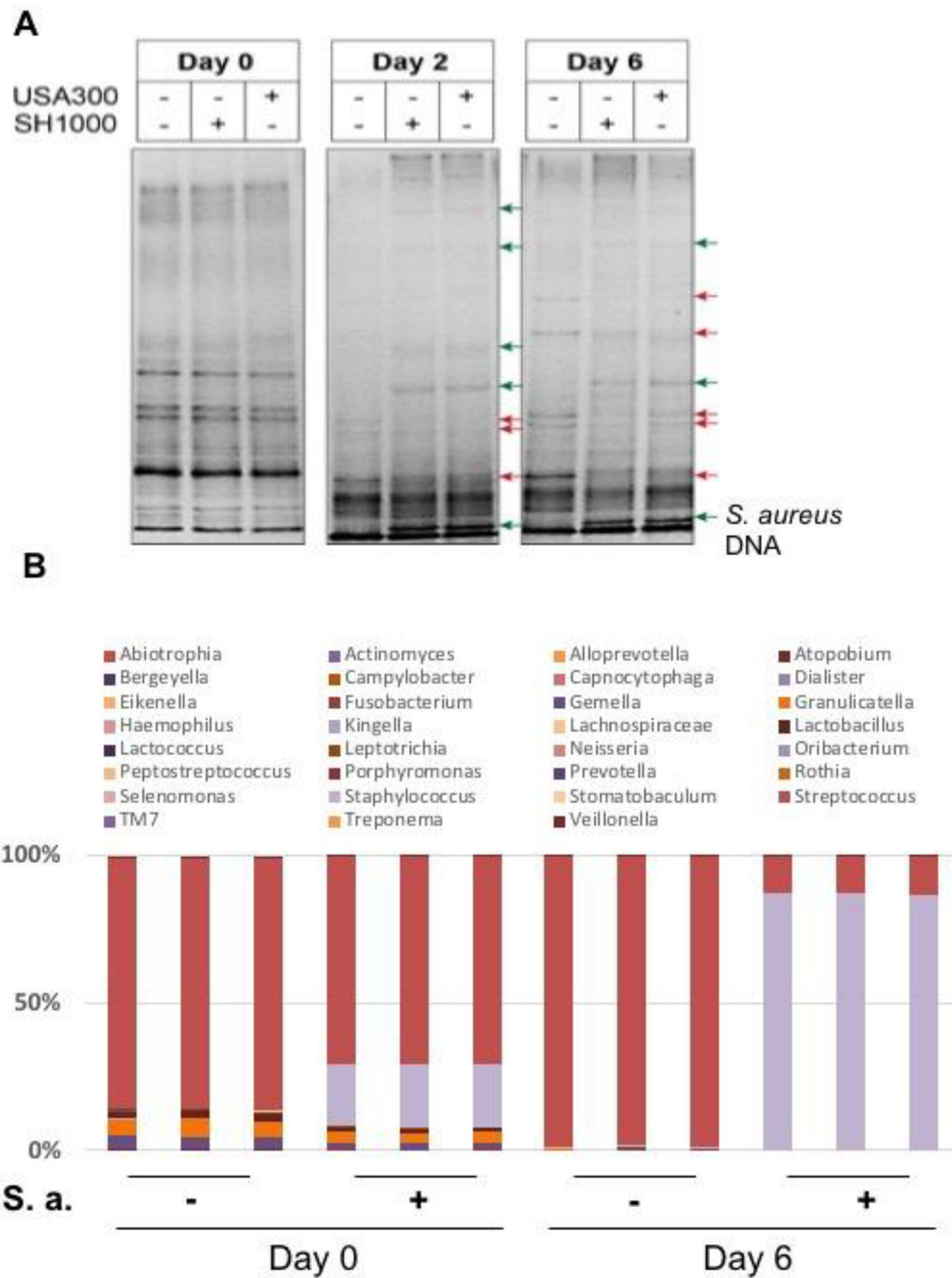


**Fig. 6).**

*S. aureus* promotes a shift in microbial composition. **(A)** DGGE analysis of microbial composition from multispecies in vitro oral biofilms +/– *S. aureus* SH1000 or USA300 at days 0, 2 and 6. Green arrows highlight bands that are more prominent in the presence of *S. aureus*, whereas the red arrows highlight bands with decreased intensity in the presence of *S. aureus*. A representative image from 3 independent experiments is shown. **(B)** Relative abundance of genus-level taxa detected from 3 biological replicates from 0 and 6-day old

biofilm +/− *S. aureus* (S.a.) SH1000 as detected by next generation sequencing of the V3 and V4 regions of the 16s rRNA gene.

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**Table 1**

Bacterial strains used in this study

| Species | Strains | Description | Source |
|---|---|---|---|
| *S. aureus* | SH1000 | *sigB+* derivative of NCTC8325-4 | [57] |
| *S. aureus* | USA300 LAC | community-acquired MRSA USA300-0114 | [58] |
| *F. nucleatum* | ATCC 23726 | ssp. *nucleatum* wildtype | ATCC |
| | *Fn0254* | ATCC 23726 *Fn0254*::pIP*0254* | [18] |
| | *Fn1526 (radD)* | ATCC 23726 *Fn1526*::pIP*1526* | [18] |
| | *Fn1554 (cmpA)* | ATCC 23726 *Fn1554*::pIP1554 | [18] |
| | *Fn1893* | ATCC 23726 *Fn1893*::pIP*1983* | [18] |
| | *Fn2047* | ATCC 23726 *Fn2047*::pIP*2047* | [18] |
| | *aim1* | ATCC 23726 *aim1*::pIPaim1 | [18] |
| *S. gordonii* | DL1 | *S. gordonii* wildtype | ATCC |
| *S. sanguinis* | ATCC 10556 | S. *sanguinis* wildtype | ATCC |
| *A. naeslundii* | ATCC 12104 | *A. naeslundii* wildtype | ATCC |
| *P. gingivalis* | 4612 | WT *P. gingivalis* | [59] |

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**Table 2**

Coaggregation of *S. aureus* with oral bacterial species

| Oral species tested | Coaggregation score |
|---|---|
| *S. gordonii* | 0 |
| *S. sanguinis* | 0 |
| *A. naeslundii* | 0 |
| *F. nucleatum* | 4 |
| *P. gingivalis* | 2 |

Wild type *S. aureus was* mixed with other species in coaggregation buffer (CAB) and allowed to aggregate for 10 mins. Coaggregation experiments were performed at three times with three biological replicates each.

Visual scoring system [60]: **0** - no visible coaggregation; **1** - Small aggregates that stay suspended; **2** - Larger aggregates that settle slowly and leave the supernatant turbid; **3**- Large aggregates that settle quickly but leave the supernatant turbid; **4** - Complete sedimentation with a clear supernatant.